Abelson Interactor Protein-1 Positively Regulates Breast Cancer Cell Proliferation, Migration, and Invasion

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Abstract

Abelson interactor protein-1 (ABI-1) is an adaptor protein involved in actin reorganization and lamellipodia formation. It forms a macromolecular complex containing Hspc300/WASP family verprolin-homologous proteins 2/ABI-1/nucleosome assembly protein 1/PIR121 or Abi/ABI-1/WASP family verprolin-homologous proteins 2 in response to Rho family-dependent stimuli. Due to its role in cell mobility, we hypothesized that ABI-1 has a role in invasion and metastasis. In the present study, we found that weakly invasive breast cancer cell lines (MCF-7, T47D, MDA-MB-468, SKBR3, and CAMA1) express lower levels of ABI-1 compared with highly invasive breast cancer cell lines (MDA-MB-231, MDA-MB-157, BT549, and HS578T), which exhibit high ABI-1 levels. Using RNA interference, ABI-1 was stably down-regulated in MDA-MB-231, which resulted in decreased cell proliferation and anchorage-dependent colony formation and abrogation of lamellipodia formation on fibronectin. Down-regulation of ABI-1 decreased invasiveness and migration ability and decreased adhesion on collagen IV and actin polymerization in MDA-MB-231 cells. Additionally, compared with control parental cells, ABI-1 small interfering RNA–transfected cells showed decreased levels of phospho-PDK1, phospho-Raf, phospho-AKT, total AKT, and AKT1. These data suggest that ABI-1 plays an important role in the spread of breast cancer and that this role may be mediated via the phosphatidylinositol 3-kinase pathway. (Mol Cancer Res 2007;5(10):1031–9)

Introduction

Breast cancer is the most common cancer in North American women and a frequent cause of female cancer mortality. Most deaths from breast cancer are due to metastases that are resistant to conventional therapies. The metastatic process involves a sequence of events, which includes detachment from the primary tumor, invasion into the vascular system, extravasation, and finally the formation of a metastatic deposit in the target organ. The migration of cancerous cells is the key step in this process. The intrinsic forces and mechanisms controlling cancer cell migration in response to various stimuli remain largely unknown.

Actin polymerization and lamellipodia formation are believed to play critical roles in cell migration during metastasis (1). Abelson interactor protein-1 (ABI-1) is an adaptor protein involved in actin reorganization and lamellipodia formation, and its function in cell spreading and migration via WASP family verprolin-homologous proteins 2 (WAVE2) is also established (2–4). Immunofluorescence of both endogenous and green fluorescent protein–tagged ABI-1 protein has indicated that ABI-1 is localized to sites of actin polymerization. These include platelet-derived growth factor–induced membrane ruffles and the tips of lamellipodia and filopodia at the cellular leading edge (5). Down-regulation of ABI-1 in cells by RNA interference indicates that ABI-1 is required for the formation of platelet-derived growth factor–induced membrane ruffles, Rac-dependent actin remodeling, cell spreading, and migration (2, 6). Further, loss of ABI-1 results in the down-regulation of WAVE2, nucleosome assembly protein 1 (Nap1), and PIR121 protein levels (3). The Rho GTPase is an important coordinator in cellular response necessary for cell migration (1). Recent investigations have shown that an ABI-1–based macromolecular complex (Hspc300/WAVE2/ABI-1/Nap1/PIR121 and/or Abi/ABI-1/WAVE2) mediates signaling transduction between Rho family proteins and the actin cytoskeleton by activating Arp2/3 (2, 3). The macromolecular complex colocalizes at the leading edge of the lamellipodia protrusion in response to numerous Rho family-dependent stimuli.

Although the function of ABI-1 has been clearly established in actin reorganization, its role in cancer progression and metastasis remains ill defined. ABI-1 is located at 10p11.2 in humans and is a homologue to the mouse abelson interactor 1 gene (7). In human acute myelogenous leukemia, ABI-1 was
found fused with *mixed lineage leukemia* gene in acute myelogenous leukemia with t(10;11)(p11.2;q23) (7-9). Chromosomal gains on 10p have been associated with a more aggressive breast cancer phenotype (10).

In this investigation, we used human breast cancer cells to determine whether ABI-1 is involved in cell proliferation and migration. Down-regulation of ABI-1 in the breast cancer cell line MDA-MB-231 resulted in decreased cell proliferation, anchorage-dependent colony formation, migration, and invasion in response to fibronectin stimulation, suggesting an important role for ABI-1 in breast cancer invasion and metastasis.

**Results**

**Expression of ABI-1, WAVE 2, PIR121, and Nap1 in Breast Cancer Cell Lines**

Nine breast cancer cell lines with varying degrees of invasiveness were selected (MDA-MB-231, MDA-MB-157, BT549, HS578T, MCF-7, T47D, MDA-MB-468, SKBR3, and CAMA1; ref. 11). Levels of ABI-1 and other members of the macromolecular complex, such as WAVE2, PIR121, and Nap1, were determined in the four highly invasive and five less invasive cell lines by Western blot analysis (Fig. 1A). An ABI-1 doublet was detected with molecular weight between 64 and 82 kDa. This likely represents two of the three isoforms of ABI-1: p68 and p72 (12). COS cells overexpressing ABI-1 were used as a positive control (data not shown). The results showed that weakly invasive breast cancer cell lines (MCF-7, T47D, MDA-MB-468, SKBR3, and CAMA1) had moderate to low expression levels of ABI-1 in contrast to highly invasive breast cancer cell lines (MDA-MB-231, MDA-MB-157, BT549, and HS578T), which showed high expression levels of ABI-1 (P < 0.01; Fig. 1B). Other members of the macromolecular complex, WAVE2 and PIR121, were expressed at significantly lower levels in the low invasive cell line group (P < 0.05 and 0.01, respectively; Fig. 1A). Nap1 showed a similar trend as well (P = 0.16). These results suggest that these proteins may have an effect on the invasive potential of breast cancer cells. ABI-1 showed the largest difference, which is consistent with our hypothesis of its involvement at several steps in the signal transduction leading to actin polymerization. The MDA-MB-231 breast cancer cell line, with one of the highest ABI-1 expression levels, was used in the subsequent studies.

**Establishment of ABI-1 Stably Down-Regulated MDA-MB-231 Clones**

pSi-ABI-1 and its control vector SiSc were used to transfect MDA-MB-231. Six stably transfected knockdown clones and 12 control clones were generated. Down-regulation of ABI-1 protein in the ABI-1 small interfering RNA (siRNA) clones was further confirmed by Western blot analysis (Fig. 2A). The expression of ABI-1 was inhibited >90% compared with control and nontransfected MDA-MB-231.

**Viability of ABI-1 Down-Regulated Cells**

To determine whether ABI-1 down-regulation affects cell viability, we did a viability assay at several time points while cells were cultured with 10% FCS and 0% FCS. No difference was observed in ABI-1 down-regulated cells compared with the parental control (Fig. 2B). This assured that the later observed changes were not secondary to apoptosis.

**ABI-1 Down-Regulation Induces Morphologic Changes in MDA-MB-231**

Down-regulation of ABI-1 had a significant effect on the morphology of MDA-MB-231 cells (Fig. 2C). The control cells exhibited mainly tripolar forms with long tapered cell processes and abundant cytoplasm. ABI-1 down-regulated cells showed less cohesion, reduced amounts of cytoplasm, and a reduced number of cell processes. Most cells were rounded with some bipolar morphology and shortened cell processes with bulbous ends.

**ABI-1 Down-Regulation Decreases Migration and Invasion in MDA-MB-231 Cells**

To study whether ABI-1 affects breast cancer proliferation, cell growth kinetics and colony formation were used. The cell growth kinetics indicated that down-regulation of ABI-1 had a significant negative effect on the proliferation of MDA-MB-231 cells resulting in decreased cell numbers (Fig. 3A). Not only was there a significant decrease (P < 0.001) in colony formation activity observed in ABI-1 siRNA–transfected MDA-MB-231 cells (Fig. 3B) but also the size of the colonies was reduced (Fig. 3B, inset).

**ABI-1 Down-Regulation Results in Cell Growth Arrest in G0-G1 and Delayed Entry into G2-M**

To understand how ABI-1 affects cell proliferation, cell doubling times were calculated in control cells and ABI-1 down-regulated cells and found to be 29.8 and 41.4 h, respectively (P < 0.01). To further investigate how ABI-1 regulates the cell cycle, cells were starved for 30 h and then cultured in 10% FCS for 21 h and the cell cycle was analyzed by fluorescence-activated cell sorting. The cell cycle analysis showed that cell growth was arrested in G0-G1 phase and entry into G2-M phase was delayed in ABI-1 down-regulated MDA-MB-231 cells. This was seen by a significant increase in cells in G0-G1 phase and a decreased number of cells in G2-M phase (P < 0.001; Fig. 3C and D). Similar results were also obtained by starving cells for 48 h (data not shown).

**ABI-1 Down-Regulation Decreases Migration and Invasion of MDA-MB-231 Cells**

We evaluated the migration of ABI-1 down-regulated MDA-MB-231 cells and their parental control cells on collagen.
IV-coated Transwell filters. There was a decrease of ~5-fold in the number of migrating cells in ABI-1 down-regulated MDA-MB-231 compared with the parental control ($P < 0.05$; Fig. 4A and B). The invasiveness of ABI-1 down-regulated cells was only ~20% of parental control cells using a Matrigel invasion assay ($P < 0.05$; Fig. 4C). We did cell counts after 48 h of starvation to estimate the proliferation rate in conditions similar to the invasion assay. The cellularity of ABI-1 down-regulated cells was reduced by 16% compared with control. This difference is too small to account for the 80% reduction in measured invasive ability. Thus, it indicates that levels of ABI-1 affect motility and invasiveness of the cancer cells.

**Phosphatidylinositol 3-Kinase/AKT Pathway Is Affected by ABI-1 Down-Regulation**

Recent evidence from different groups suggests that AKT1 may negatively regulate migration both in breast cancer cells and in the normal mammary cell line MCF 10A (13, 14). Considering this reported AKT involvement in cell migration and our results in ABI-1 down-regulated MDA-MB-231 cells, we decided to assess phosphatidylinositol 3-kinase (PI3K)/AKT pathway proteins in ABI-1 down-regulated cells at basal culture conditions. Immunoblotting with antibodies to phospho-PDK1, phospho-Raf, phospho-AKT, and total AKT showed that ABI-1 down-regulation resulted in a constant striking decrease in these proteins. Further AKT isoform analysis showed that it was AKT1 rather than AKT2 that was abolished in ABI-1 down-regulated cells (Fig. 5A).

**ABI-1 Down-Regulation Results in Reduced Levels of WAVE2, PIR121, and Nap1**

ABI-1 is a member of the macromolecular complex Hspc300/WAVE2/ABI-1/Nap1/PIR121, which is unstable without ABI-1 (3). Because its role in breast cancer is unclear,
we assessed the levels of the complex members in ABI-1 down-regulated cells in comparison with their parental controls. There was a significant reduction of WAVE2, PIR121, and Nap1 levels in ABI-1 down-regulated cells (clones 9, 11, and 15; Fig. 5B).

**Discussion**

ABI-1, being an adaptor protein, is involved in actin reorganization and lamellipodia formation by forming a macromolecular complex containing Hspc300/WAVE2/ABI-1/Nap1/PIR121 or Abl/ABI-1/WAVE2 to mediate signal transduction from Rac to the Arp2/3 complex (15). Currently, most studies have focused on the interacting partners of ABI-1 and their roles in actin dynamics (2, 16, 17). There is relatively little information on the role of ABI-1 in cancer cell motility, an important step in metastasis. As actin polymerization is involved in cell migration, it is important to explore whether ABI-1 plays a role in cell migration. It is also not clear if ABI-1 affects cell proliferation. Thus, we investigated whether ABI-1 has a role in proliferation, formation of lamellipodia, and cell migration in breast cancer cell lines. To the best of our knowledge, this is the first investigation of the expression and function of ABI-1 in breast cancer cells.

As a first step to understanding the involvement of ABI-1 in cancer cells, we measured expression levels of ABI-1 in nine cell lines.
different breast cancer cell lines. The results showed that highly invasive breast cancer cells had much higher expression levels of ABI-1 than weakly invasive breast cancer cells. To further elucidate the functions of ABI-1 in breast cancer cells, we down-regulated ABI-1 expression in MDA-MB-231 cells by RNA interference. Our results showed that knockdown of ABI-1 expression not only abrogated the formation of lamellipodia but also inhibited MDA-MB-231 cell adhesion on collagen IV. These results support the previously reported data on the involvement of ABI-1 in the formation of lamellipodia.

Actin reorganization plays a critical role in leading cell migration. A previous report showed that ABI-1 down-regulation in B16F1 melanoma cells impairs migration (2). More recently, data from different groups have suggested that the ABI-1/
WAVE2 complex is crucial for actin dynamics and migration in macrophages and T cells in response to various stimuli (6, 18). As expected, our results showed that the migration and invasive ability was significantly decreased in ABI-1 down-regulated MDA-MB-231 cells compared with control cells (Fig. 4A-C).

In our model system, multiple levels of regulation may contribute to this phenotype. First, the loss of the direct effect of Hspc300/WAVE2/ABI-1/Nap1/PIR121 complex on its downstream target Arp2/3 complex and actin may affect cell migration. Second, ABI-1 can affect PI3K and AKT1 through Rac and Cdc42, which results in decreased migration ability (Fig. 6). Lastly, decreased adhesion to extracellular matrix, which we found to be the result of ABI-1 suppression, may also have a role in inhibiting the migration and invasion.

Although the role of ABI-1 has been clearly established in actin reorganization, its roles in cell growth and tumorigenesis remain controversial. Some reports have indicated that overexpression of ABI-1 inhibits growth and transforming activity of v-Abl in NIH3T3 cells as well as epidermal growth factor-induced and v-Abl-induced activation of extracellular signal-regulated kinase in 293T cells (12, 19-22), which suggest a potential negative regulatory role of ABI-1 on epidermal growth factor–mediated signaling. Other studies have found that loss of ABI-1 may play a role in human prostatic adenocarcinoma and the oncogenesis of Bcr-Abl–positive leukemia (23, 24). However, experiments with Drosophila suggest that both fly and human ABI-1 can enhance the ability of Abl to

**FIGURE 4.** The migration and invasion ability of breast cancer cells is positively related to the ABI-1 levels. A. Representative photos of migration assay. Magnification, ×100. B. ABI-1 down-regulation in MDA-MB-231 results in decreased migration in comparison with their parental control cells. C. ABI-1 down-regulation in MDA-MB-231 results in decreased invasion compared with their parental control cells. The total number of migrating or invasive cells was counted in the entire field. Results are expressed as percentage of parental control cells. B and C. Columns, mean of three independent experiments in siRNA clones 2, 9, and 11; bars, SE. *P < 0.05. Viable cell count after 48-h starvation was done to estimate the effect of proliferation rate in similar conditions on invasion and migration assays (ABI-siRNA was 84% of control; mean, 0.38 × 10⁶ and 0.32 × 10⁶ cells/mL for control and siRNA clones, respectively). It showed that the observed change in migration ability was not due to decreased proliferation.

**FIGURE 5.** A. ABI-1 down-regulation in MDA-MB-231 cells results in decreased PI3K/AKT pathway proteins compared with their parental control cells. Cells cultured at normal conditions were lysed in Cytobuster, and 20 μg total protein was separated by a 4% to 12% SDS-NuPage gradient gel and transferred to Hybond-P polyvinylidene difluoride membrane and blotted with the antibodies as indicated (representative photos, numbers shown are means of three independent experiments in siRNA clones 9 and 15). P-AKT, phosphorylated AKT; P-PDK1, phosphorylated PDK1; P-Raf, phosphorylated Raf. B. Reduced expression of the Hspc300/WAVE2/ABI-1/Nap1/PIR121 complex proteins in ABI-1 down-regulated cells. Total protein (25 μg) loaded and blotted as above (representative photos, three independent experiments in siRNA clones 9, 11, and 15).
ABI-1 Regulates Proliferation, Migration, and Invasion

As an adaptor protein, ABI-1 is involved in several signaling pathways by forming Hspc300-WAVE2-ABI-1-Nap1-PIR121 complexes (3, 28). We observed decreased levels of WAVE2, PIR121, and Nap1 in the cell lines with low ABI-1 expression (Fig. 1A). These proteins were also down-regulated in ABI-1 knockdown cells (Fig. 5B). These data show that the Hspc300/WAVE2/ABI-1/Nap1/PIR121 complex is dependent on ABI-1 in breast cancer cell lines, which is consistent with previous reports in other systems (3).

A direct interaction between p85 and ABI-1 has been suggested to be necessary for ABI-1-dependent Rac activation (28). Therefore, we were not surprised that levels of the PI3K/AKT pathway proteins we observed may be the consequence of alterations in multiple signal transduction pathways, and conversely, it may also regulate the functions of other pathways.

In summary, we have shown differential ABI-1 expression levels in several breast cancer cell lines associated with varying phenotypes. Down-regulation of ABI-1 in MDA-MB-231 resulted in decreased adhesion, cell proliferation, migration, and invasion as well as abrogated lamellipodia formation. Compared with control parental cells, ABI-1 siRNA–transfected MDA-MB-231 cells showed lower levels of PI3K/AKT pathway members, which may contribute to the decreased proliferation, migration, and invasion we observed. Whether this surprising phenotype is a cell type–specific effect or is universal in cancer cells remains to be determined. We have detected high expression of ABI-1 in some pancreatic and prostate cancer cell lines and further investigation is under way. Work on other breast cancer cell lines is also ongoing. Nevertheless, these results suggest that ABI-1 could have a pivotal role in breast cancer progression. Further in vivo and clinical studies are required to determine the prognostic and/or predictive value of ABI-1 in human breast cancer.

Materials and Methods

ABI-1 siRNA Vectors and Antibodies

pAV-ABI-1 siRNAs, spanning nucleotides 169 to 187 (pAV169) and 197 to 215 (pAV197), were kind gifts from Dr. G. Scita (Italian Foundation for Cancer Research, Institute of Molecular Oncology Foundation, Milan, Italy). Rabbit polyclonal anti-Nap1 and anti-PIR121 were from Dr. T. Stradal (National Research Center for Biotechnology, Braunschweig, Germany). ABI-1 siRNA vectors (pSi-ABI-1), scrambled control vectors (pSiSc), and rabbit anti-human ABI-1 were as described previously (2). Antibodies against phospho-PDK1, phospho-Raf, phospho-AKT, and AKT1 and AKT2 were purchased from Cell Signaling Technology. Goat polyclonal anti-WAVE2 was from Santa Cruz Biotechnology. Rhodamine-coupled phallolidin was obtained from Invitrogen Life Technologies. Glyceraldehyde-3-phosphate dehydrogenase was obtained from Abcam.

Cell Culture and Transfections

MDA-MB-231 cell line (American Type Culture Collection) was maintained in DMEM supplemented with 10% FCS (Hyclone), 100 μg/mL streptomycin, and 100 units/mL penicillin. Cells were transfected with pSi-ABI-1 (or pAV-ABI-1 siRNAs) using LipofectAMINE 2000 (Invitrogen Life Technologies). Cells were cultured in 10-cm dishes until 70% to 80% confluent and transfected with 5 μg of pSi-ABI-1. Forty-eight hours after transfection, cells were placed into selection medium containing 2 mg/mL G418 (Invitrogen Life Technologies) and individual G418-resistant colonies were screened by Western blotting. Control cells were transfected with pSiSc in decrease levels of AKT through reduced activation of these proteins. It is also believed that there is a positive feedback loop between adhesion and Rac activation, which stimulates the recruitment and/or activation of PI3K at the plasma membrane (Fig. 6; ref. 30). Therefore, the decreased levels of the PI3K/AKT pathway proteins we observed may be the consequence of alternations in multiple signal transduction pathways, and conversely, it may also regulate the functions of other pathways.

FIGURE 6. ABI-1 interactions with the Sos1 and WAVE2 complexes and their relationship to Rac, actin polymerization, and PI3K. Actin polymerization has a positive feedback effect on PI3K and Rac. RTK, receptor tyrosine kinase; EGF, epidermal growth factor; PDGF, platelet-derived growth factor; PI3K, phosphatidylinositol 3-kinase.
the same manner. ABI-1 down-regulation was confirmed by showing at least a 90% reduction in signal intensity by Western blotting compared with control pSiSc-transfected clones. pAV-ABI-1 siRNAs were cotransfected with pSiSc for G418 selection, which resulted in a similar phenotype to pSi-ABI-1.

**Cell Viability Assay**

Cells were cultured in 10% FCS and free of FCS for different time points and harvested. Cell viability assay was done with Vi-CELL XR cell viability analyzer with Vi-CELL XR 2.03 software (Beckman Coulter).

**Cell Proliferation and Clonogenic Assay**

ABI-1 down-regulated MDA-MB-231 (pSi-ABI-1, clones 2, 9, 11, and 15) and control MDA-MB-231 cells (pSiSc) were plated at a density of 1 × 10^4 per well of six-well plates in triplicate. Cells were harvested at days 1, 3, and 7, respectively, and counted with a Beckman Coulter cell counter (Beckman Coulter Canada). For anchorage-dependent colony formation, ABI-1 down-regulated MDA-MB-231 and control MDA-MB-231 cells were plated at a density of 100 per well of six-well plates in triplicate and cultured for 14 days in the presence of G418. Cells were stained with 0.1% Coomassie blue (Bio-Rad) in 30% methanol and 10% acetic acid. Colony-forming efficiencies after plating (≥50 cells per colony) were evaluated (colony number/total plated cell numbers).

**Flow Cytometry Analysis**

ABI-1 siRNA (clones 2, 9, 11, and 15) and control MDA-MB-231 cells were plated at a density of 3 × 10^5 in 6-cm dishes in triplicate and cultured overnight in medium containing 10% FCS. The cells were subjected to starvation with medium free of FCS for 30 h followed by culturing in medium containing 10% FCS for 24 h. Cells were collected, counted, washed with PBS, and fixed with ice-cold 80% ethanol for 60 min. They were washed with PBS thrice and permeabilized with 0.2% Triton X-100 for 5 min and then stained with 1 mL propidium iodide/RNase staining buffer (BD Biosciences) for 60 min. Cells were acquired with a FACScan flow cytometer and CellQuest software (Becton Dickinson). The cell cycle was analyzed with ModFit II LT software (Verity Software House, Inc.).

**Transwell Migration and Invasion Assay**

Transwell migration and invasion assays were done as described previously (31). In brief, ABI-1 siRNA (clones 2, 9, and 11) and their parental control MDA-MB-231 were serum starved for 24 h, harvested, and resuspended in medium containing 2% bovine serum albumin. Cells (5 × 10^4) were added to the top chambers of 24-well Transwell plates coated with 7.5 μg collagen IV or Matrigel (8-μm pore size; BD Biosciences), and medium containing 5 μg/mL fibronectin was added to the bottom chambers. Cells were incubated for 48 h at 37°C. The cells were fixed with 0.1% glutaraldehyde-PBS for 20 min, rinsed briefly with double-distilled water, and stained with 0.2% crystal violet for 1 h. The filters were washed thoroughly with double-distilled water. Nonmotile cells on top of each filter were removed by wiping with cotton swabs. The number of migrating cells or invasive cells was counted with a Beckman Coulter cell counter (Beckman Dickinson), incubated for 1 h at 37°C, and then rinsed with PBS four times. The remaining cells in each well were fixed in 10% buffered formalin for 20 min at room temperature and washed with PBS, and the entire field was counted with a stereomicroscope. All images were recorded and analyzed using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.).

**Western Blotting**

Cells cultured at normal conditions were lysed in CytoBuster protein extraction reagent (Novagen) containing kinase and phosphatase inhibitors. The supernatants were collected by microcentrifugation at 10,000 × g for 5 min at 4°C. For ABI-1, WAVE2, Nap1, and PIR121 detection, 25 μg total protein was used, and for all other target markers 20 μg total protein was separated by a 4% to 12% SDS-NuPage gradient gel (Invitrogen Life Technologies). Immunoblot analysis was done using standard methods. Images were scanned by Bio-Rad scanner and quantified by Quantity One software (Bio-Rad).

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**Spreading and Adhesion Assays**

Cells were plated on a 10 μg/mL fibronectin precoated glass coverslip in medium free of FCS for 6 to 18 h, fixed with 3.7% formaldehyde/PBS solution, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. Cells were stained for filamentous actin with rhodamine-coupled phalloidin (1:40 dilution) for 30 min, washed briefly with PBS, and mounted to standard slides with antifading mounting medium containing 4',6-diamidino-2-phenylindole. Images were acquired with a Zeiss LSM 510 confocal microscope.

For the cell adhesion assay, 5 × 10^4 cells were plated in each well of 24-well plates coated with collagen IV (Becton Dickinson), incubated for 1 h at 37°C, and then rinsed with PBS four times. The remaining cells in each well were fixed in 10% buffered formalin for 20 min at room temperature and washed with PBS, and the entire field was counted with a stereomicroscope. All images were recorded and analyzed using Image-Pro Plus software (version 6.0; Media Cybernetics, Inc.).

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